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(71) Applicant *(for all designated States except US)*: DIAMYD THERAPEUTICS AB [SE/SE]; Djugårdsbrunnsvägen 54, S-115 25 Stockholm (SE).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: ROBERTSON, John [GB/SE]; Karolinska Sjukhuset M:300, S-171 76 Stockholm (SE).

(74) Agents: BERGSTRAND, Mikael et al.; Albihns Patentbyrå Stockholm AB, P.O. Box 3137, S-103 62 Stockholm (SE).

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(54) Title: USE OF A ZWITTERIONIC DETERGENT/SURFACTANT FOR PURIFYING GLUTAMIC ACID DECARBOXYLASE (GAD) AND A COMPOSITION CONTAINING GAD

(57) Abstract

The extraction and purification of glutamic acid decarboxylase is improved by using buffer solutions containing zwitterionic detergents/surfactants preferably according to the following chemical formula: $C_yH_{2y+1} - N^+(CH_3)_2 - C_xH_{2x} - SO_3^-$ wherein x is an integer from 1 to 5 and y is an integer from 2 to 20.

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Use of a zwitterionic detergent/surfactant for purifying glutamic acid decarboxylase (GAD) and a composition containing GAD.

The present invention relates to a novel and improved method of producing glutamic acid decarboxylase. In particular the invention relates to a novel procedure for purifying the enzyme from recombinant cells engineered to produce the enzyme or from native cells or tissues containing it.

Background of the invention

Insulin-dependent diabetes mellitus (IDDM) results from the progressive autoimmune destruction of insulin-producing beta cells in the pancreas and, consequently, the inability to regulate blood sugar levels. This (otherwise fatal) condition is currently managed by treatment with exioguously-derived insulin. IDDM patients, however, often have but a lowered life expectancy and quality due to long-term complications. Accordingly, there is considerable interest in defining a therapeutic to prevent IDDM.

One therapeutic strategy is aimed at preventing autoimmune destruction of pancreatic beta cells via a process of "tolerisation" whereby treatment with specific autoantigens can result in immunological "re-programming" to re-establish appropriate "self" from "non-self" distinction.

Antibodies to the enzyme: glutamic acid decarboxylase (GAD) have been shown to precede the onset of IDDM (Baekkeskov et al. (1987) J. Clin. Invest. 79:926-934, Baekkeskov et al. (1990) Nature 347:151-156, all of which are incorporated by reference) and, furthermore, to provide a highly predictive marker for future development of this disease.

Although this enzyme was known to be important in neurotransmission (in synthesis of the major neuroinhibitor: gamma amino butyric acid) its exact role in the aetiology of IDDM has remained unclear. However, the clinical presence of GAD antibodies and GAD-reactive T-cells identifies this protein as an autoantigen and implicates its involvement in the autoimmune destruction of pancreatic beta cells - the patho-aetiological "hallmark" of IDDM.

Despite the existence of 2 isoforms of this enzyme having molecular weights of 65 kDa and 67 kDa respectively(GAD65 and GAD67), data only convincingly support the involvement of the former (GAD65) in IDDM. Both forms are very rare in all tissues and, accordingly, this protein has been difficult to purify. Manufacture of the recombinant form of the human protein (rhGAD65), however, has become possible after cloning of the cDNA for human GAD65 (hGAD65) (Karlsen et al. 1991 Proc. Natl. Acad. Sci. 88:8337-8341; Bu et al. 1992 Proc. Natl. Acad. Sci. 89:2115-2119, all of which are incorporated by reference).

Of central relevance to the present context was the finding reported by 2 separate groups (Kaufman et al. 1993 Nature 366:69-72; Tisch et al. 1993 Nature 366:72-75, all of which are incorporated by reference) that a single treatment with rhGAD65 was sufficient to induce immunological tolerance and thereby prevent pancreatic beta cell destruction (and IDDM) in the "non-obese diabetic" murine model for IDDM.

Accordingly, and in conjunction with promising preliminary reports regarding tolerisation therapies (with other autoantigens, for multiple sclerosis and rheumatic arthritis) considerable interest has been generated regarding the possibility of preventing human beta cell destruction (i.e. IDDM) by the administration of rhGAD65 to IDDM patients or otherwise healthy individuals "at-risk" for IDDM.

Extraction and purification of the 65K isoform of glutamic acid decarboxylase from animal tissues has been hindered due to the low levels of the enzyme present in all species examined. Despite this, work in the early 1960s had begun on stability and partial purification from several mammalian species. These included purification procedures from brain of:

mouse (Susz et al. 1966 Biochemistry 5(9):2870-2877) or
human (Blinderman et al. 1978 Eur. J. Biochem. 86:143-152) or
pig (Spink et al. 1985 Biochem. J. 1985 231:695-703) or
rat (Wu et al. 1985 Methods Enzymol. 113:3-10;
Martin et al. 1990 J. Neurochem. 55(2):524-532) or
chicken (Gottlieb et al. 1986 83:8808-8812) or
cow (Wu 1982 Proc. Natl. Acad. Sci. USA 79:4270-4274),
all of which are incorporated by reference.

It is to be stressed that, at the time of these studies, the presence of 2 forms (i.e. GAD65 and GAD67) was not confirmed. Accordingly, the single publication reporting purification of human GAD (Blinderman et al. 1978) reports the molecular size of that GAD as 67,000 - which thereby implies this as the 67 kDa isoform (hGAD67) and not hGAD65. This is an important consideration here because subsequent studies have shown considerable differences in physicochemical properties between these 2 forms. Consequently, few scientific reports regarding purification of GAD from tissues are of direct relevance to the properties of the recombinant human form of GAD65 under consideration here.

After the first cDNA cloning of the human enzyme (Karlsen et al. 1991 Proc. Natl. Acad. Sci. 88:8337-8341) production of the recombinant form (rhGAD65) was facilitated and, to date, has included use of the following prokaryotic and eukaryotic cells for expression:

bacteria	Kaufman et al. 1992 J. Clin. Invest. 89:283-292 and 1993 Nature 366:69-72
mammalian	Hagopian et al. 1993 J. Clin. Invest. 91(1):368-374; Shi et al. 1944 J. Cell Biol. 124(6):927-934
insect	Christgau et al. 1992 J. Cell Biol. 118:309-320; Tisch et al. 1993 Nature 366:72-75; Mauch et al. 1993 J. Biochem. 113(6): 699-704; Moody et al. 1995 Diabetologia 38; 14-23; all of which are incorporated by reference.

The initial cloning of rhGAD65 reported by Karlsen et al. 1991 Proc. Natl. Acad. Sci. USA 88:8337-8341 involved hGAD67-derived oligonucleotide probing of a human islet cDNA library, and that by Bu et al. 1992 Proc. Natl. Acad. Sci. 89:2115-2119 used a 180 bp fragment of the rat GAD65 cDNA sequence (Erlander et al. 1991 Neuron 7:91-100 all of which are incorporated by reference) to probe a human hippocampus cDNA library.

Cloning of rhGAD65 cDNA into baculovirus by Christgau et al. 1992 J. Cell Biol. 118:309-320 used that same rhGAD65 cDNA isolated by Bu et al. 1992 (i.e. provided as a gift from Professor Tobin), and that reported by Moody et al. 1995 Diabetologia 38; 14-23 used the cDNA isolated by Karlsen et al. 1991 (i.e. provided as a gift by Professor Lernmark).

The procedure reported by Mauch et al. 1993 J. Biochem. 113(6):699-704, incorporated by reference, used a (Taq polymerase) PCR-based approach using hGAD67 DNA primers to screen a human pancreas cDNA library.

Only the "Triton" class of non-ionic detergents have been reported in the scientific literature for extraction and purification of GAD. Triton X-114 in the extraction and/or partial purification of GAD has been reported by:

Baekkeskov et al. 1990 Nature 347:151-157
Christgau et al. 1992 J. Cell. Biol. 118(2):309-320
Shi et al. 1994 J. Cell Biol. 124(6):927-934
Solimena et al. 1994 J. Cell Biol. 126:?-? ??
Moody et al. 1995 Diabetologia 38:14-23.
all of which are incorporated by reference.

The method employed follows from previous publication concerning use of this detergent in a general method for extraction and separation of hydrophobic proteins (Bordier 1981 J. Biol. Chem. 256(4):1604-1607).

Use of the non-ionic detergents: Triton X-100, Triton X-114 and n-octylglucoside, as well as the ionic detergent: sodium dodecyl sulphate - are also specified in WO95/04137

The use of Tritons, however, has been found to have inherent problems, including:

1. Tritons are not chemically-defined (thereby imposing difficulties regarding definition of a GMP-compliant process)

2. Triton-solubilised proteins (including rhGAD65) are not easily dialysed or ultrafiltered. Accordingly, the inclusion of Triton X-114 is likely to complicate downstream processing of rhGAD65.

3. Triton X-114-extracted insect cells expressing rhGAD65 have been found to have high viscosity - resulting in prolongation of chromatographic application

times (by imposing extremely low chromatographic flow-rates). Consequently, the use of Triton X-114 extends run times considerably, and its inclusion is assessed by us as not enabling a cost-effective process.

4. The Triton X-114 extraction method was discovered by us to result in the co-extraction of the baculoviral glycoprotein "gp64" with rhGAD65. Furthermore, as these two proteins are not readily distinguishable (they co-migrate in SDS-PAGE) it is considered advantageous to avoid their co-purification at an early purification stage.

5. The method of Bordier (1981) requires the preliminary preparation of water-saturated Triton X-114 (during a "pre-condensation" step) prior to its use as a protein extractant. Inclusion of agents to minimise peroxide formation may also be required. Accordingly, use of Triton X-114 as extractant will necessitate inclusion of an additional, preliminary step prior to its use in a manufacturing process.

6. The Triton X-114 extraction method of Bordier (1981) involves 1-3 condensation steps - whereby the detergent-rich phase containing solubilised hydrophobic proteins (i.e. including rhGAD65) is separated from hydrophilic proteins in the water-rich phase (by lowering the temperature below the "cloud point" of 20°C). This method, therefore, is not readily-scaleable, and inclusion of Triton X-114 condensation steps for extraction and partial purification will significantly lengthen a commercial manufacturing process.

With regard to the use of n-octylglucoside, this is regarded as an inferior detergent due its high cost and because it readily allows microbial growth, and is not therefore conducive to a scaled-up manufacturing process.

Regarding use of sodium dodecyl sulphate, it is relevant to mention that this is reported (in WO95/04137) as effective at 0.1%, but completely eliminates rhGAD65 enzyme activity at 1.0%.

Hence, there is a need to find alternative detergents in order to overcome the above mentioned problems and to achieve an overall improved extraction process.

Summary of the invention

Now, it has turned out that by using a zwitterionic detergent/surfactant preferably according to the following chemical formula:

$$C_yH_{2y+1} - N^+(CH_3)_2 - C_xH_{2x}-SO_3^-$$

wherein x is an integer from 1 to 5 and y is an integer from 2 to 20, such as N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate, surprisingly good purification results are achieved at the same time as most of the above mentioned problems are solved. The zwitterionic detergent is preferably used both when extracting proteins from GAD-containing cells and during subsequent purification steps, such as ion exchange chromatography. In addition, the use of the zwitterionic detergent/surfactant surprisingly provides an internal reference check of IEX resolution as it separates another, ca. 65kDa baculoviral chitinase protein from rhGAD 65. This chitinase protein is different from the earlier mentioned baculovirus 64 kDa glycoprotein and is likely to remain a contaminant in rhGAD 65 preparations where Triton or Octylglucoside have been used.

Detailed description of the invention

As already mentioned, the present invention relates to using a zwitterionic detergent/surfactant, preferably of a type disclosed in the appended claims 4-5, in a process for purifying glutamic acid decarboxylase (GAD). The invention also relates to compositions obtainable by the process, which compositions comprises GAD and a zwitterionic detergent/surfactant. These compositions are suitable as pharmaceutical for treating IDDM, cancer, etc. They can also be used as vaccine compositions against autoimmune diseases such as IDDM, rheumatoid arthritis etc. Moreover, the compositions can be used for determining the presence of antibodies against glutamic acid decarboxylase (GAD). Such assays are valuable when diagnosing autoimmune diseases.

Finally, the compositions are suitable as pharmaceutical agents for treating Stiff Man Syndrome and Graves' disease, They can also be used in vaccine compositions against Coxsackie virus, polio, mumps, parotis and rubella.

Despite evidence for the inhibitory activity of phosphate (Roberts & Simonsen 1963 Biochem. Pharmacol. 12:113-134), this buffer has subsequently been used in several GAD purifications, including GAD from human brain (Blinderman et al. 1978 Eur. J. Biochem. 86:143-152), pig brain (Spink et al. 1985 Biochem. J. 1985 231:695-703), rat brain (Martin et al. 1990 J. Neurochem. 55(2):524-532), mouse brain (Wu et al. 1973 J. Biol. Chem. 248(9):3029-3034), and bovine brain (Wu et al. 1982 Proc. Natl. Acad. Sci. USA 79:4270-4274 all of which are incorporated by reference).

Alternatively, HEPES buffer at 50 mM and pH 7.2 has been successfully used in studies of GAD from murine brain (Meeley & Martin (1983) Cellular & Molec Neurobiol. 3(1):55-68) and pig brain (Porter & Martin (1988) J. Neurochem. 51(6):1886-91). HEPES has also been used for initial fractionation of rhGAD65

from insect cells (10 mm used by Christgau et al. 1992 J. Cell. Biol. 118(2):309-320; all of which are incorporated by reference).

Reducing agents are frequently used for stabilisation during extraction and purification of proteins, and use of DTT has been recommended for protein stabilisation during detergent extraction (Hjelmeland and Chrambach 1984 Methods Enzymol. 114:305-318). We note that a high concentration of DTT (50 mM) has been used with Zwittergen 3-12 for purification of the human interferon "Betaseron" (Russel-Harde et al. 1995 J. Interferon & Cytokine Res. 15:31-37, all of which are incorporated by reference).

A reducing agent is included to minimise inter- and intra-molecular disulphide bridge formation by maintaining thiol groups (cyteine residues) in their reduced state. As our prior analysis of the published hGAD65 cDNA sequence readily identified 15 cysteines present in rhGAD65, the rationale for inclusion of high concentrations of a reducing agent is obvious.

The use of the reductants for GAD stabilisation was reported early on: reduced glutathione, cysteine, and beta-mercaptoethanol were found to stabilise murine GAD (Roberts & Simonsen 1963 Biochem. Pharmacol. 12:113-134) and reduced glutathione subsequently used to prepare GAD from bovine brain (Wu 1982 Proc. Natl. Acad. Sci. USA 79:4270-4274 all of which are incorporated by reference).

Pyridoxal-5-phosphate (PLP) has previously been shown to stabilise GAD (Roberts & Simonsen (1963) Biochem Pharmacol 12:113-134) and subsequently used to purify murine brain GAD (0,2 mM used by Wu et al. 1973 J. Biol. Chem. 248(9):3029-3034). This stabilising agent has also used (at 0.5 mM) for extraction of GAD from human brain (Blinderman et al. (1978) Eur. J. Biochem. 86:143-152); for rat brain (used at 0,2 mM by Wu et al. (1985) Methods Enzymology 113:3-10); and for bovine brain (used at 0,2 mM by Wu (1982) Proc. Natl. Acad. Sci. USA

79:4270-4, all of which are incorporated by reference). Use of 0.2-1.0 mM PLP is also mentioned in WO95/04137.

The use of protease inhibitors are often mandatory in the extraction and purification of recombinant proteins from eukaryotic expression systems. However, certain of these (e.g. APMSF) are highly toxic and their inclusion is to be avoided in a commercial process. In addition, the separate addition of individual protease inhibitors prior to cell extraction was unattractive during the design of our process, and has been avoided by us by the addition of single tablets of a pre-made "cocktail" of complete protease inhibitors.

The invention will now be described with refrence to the enclosed figures, in which:

Fig. 1 shows a Western blot disclosing the presence absence of GAD in a cell homogenate and in the supernatant as well as in the pellet after the cell homogenate has been ultracentrifugated. The cell homogenate has been prepared according to the present invention.

Fig 2 discloses the result of an ion exchange chromatography purification step according to a preferred embodiment of the present invention;

Fig 3 illustrates the degree of purification after ion exchange chromatography by showing an electrophoresis gel in which lane 1 corresponds to unpurified protein extract and lanes 2-6 correspond to different dilutions of the purified rhGAD65 enzyme; and

Fig 4 discloses rhGAD65-specific immunoreactivity by Western blotting.

EXAMPLE 1

Extraction of GAD from baculovirus-infected Sf9 insect cells

Baculoviral expression of rhGAD65 in Sf9 insect cells

Recombinant cells expressing GAD were prepared as disclosed in Christgau et al., 1992, J. Cell. Biol. Vol. 48: 309-320, 1.2 x 10^{10} pelleted (i.e., centrifuged) (in 1L perfusion culture) and re-suspended Sf9 cells were washed once in ice-cold PBS + 5 mM EDTA. The final washed pellet volume obtained was 40 mL, and contained in one 50 ml Falcon tube.

EQUIPMENT

Ultracentrifuge
50.2 Ti fixed angle rotor (Beckman)
Tubes, polycarbonate

CHEMICALS

"Complete" protease inhibitor cocktail tablets (Boehringer Mannheim 1697498)
Pyridoxal-5-Phospate (Janssen/Chemicon 2281722)
DTT (Sigma D-5545)
Zwittergent 3-12, N-Dodecyl-N,N-dimethyl-3-ammonio-1-propansulphonate (Boehringer Mannheim 1112724)
HEPES (Sigma H-3375)
0.5 M EDTA (Sigma E-7889)
NaCl (Merck 1.06404.1000)

REAGENTS

1 M HEPES stock

(pH 7.2)

HEPES 119.15 g was dissolved in 350 ml sterile distilled water. pH was adjusted to 7.2. The volume was adjusted to 500 ml and the buffer was passed through a sterile filter, 0.2 microns.

Zwit Extraction buffer (ZEB) 2x

(80 mM HEPES, 10 mM EDTA, 20 mM DTT, 0.1 mM Pyridoxal-5-phospate, 4% Zwittergent 3-12, pH 7.2)

Stock solution

Sterile distilled water	22 ml
1 M HEPES stock	2 ml
0.5 M EDTA	0.5 ml
Zwittergent 3-12	1 g

The following was added before use:

DTT	75 mg
Pyridoxal-5-phosphate	0.62 mg
"Complete" tablets	2 tablets

25 ml of ZEB 2x was obtained.

ZEB 1x

(40 mM HEPES, 5 mM EDTA, 10 mM DTT, 0.050 mM Pyridoxal-5-phospate, 2% Zwittergent 3-12, pH 7.2)

Stock solution:

Sterile distilled water	118.75 ml
1 M HEPES stock	5 ml
0.5 M EDTA	1.25 ml
Zwittergent 3-12	2.5 mg

The following was added before use:

DTT	188 mg
Pyridoxal-5-phospate	1.55 mg
"Complete" tablets	3 tablets

125 ml of ZEB 1x was obtained.

The extraction was carried out according to the following protocol:

All extraction steps were - whenever possible - conducted in water ice.

1. Remove the 50 ml Falcon containing this Sf9 pellet (nb. is 40 ml) from storage at below - 70°C to temporary storage in dry ice.

2. Remove this tube from dry ice, and add 25 ml of the 2x Zwit Extraction Buffer (2x ZEB, see this protocol for composition) to the frozen pellet.

3. Allow to partially thaw (to dislodge pellet) and immediately transfer to (pour into a pre-chilled Dounce (nb. Dounce will hold up to 70 ml).

4. Dounce (on ice) using 20 strokes after the pellet has been completely disrupted.

5. Pour homogenate into a clean beaker (on ice).

6. Adjust the 65 ml (was 70 ml) of this initial homogenate to 110 ml (final) by addition of (40 m) of 1xZEB, and mix by swirling.

7. Remove 2, successive volumes (2 x 55 ml) of homogenate and repeat Dounce (5 strokes each), then transfer to Falcons (3 x 40 ml) - after removal of 10 x 0.025 ml aliquots to pre-marked microfuges. Homogenate is now ready for ultra-centrifugation.

8. Spin for 30 min at 100,000x g at 4°C (use 30,000 rpm in 50.2 Ti fixed angle rotor in L5.50) - in (4) tubes containing (27.5) ml in each.

rotor used:	50.2 Ti
tubes used:	polycarbonate
speed (rpm):	30,000 for 100,000x g

9. Decant clear supernatant from each ultraC tube (by pouring), and transfer to 3 x 50 ml pre-marked Falcon tubes.

This provides 90 ml of extract.

10. Remove/save 10 x 0.025 mL aliquots of this supernatant fraction, and store at -70°C.

11. Thoroughly re-suspend one debris pellet in 27.5 ml ZEB (using Dounce), and remove 5 x 0.05 mL aliquots to pre-marked microfuge tubes (for confirmation of extraction efficiency only; discard remaining re-suspended pellet).

12. Freeze/store ALL fractions at -70°C.

The results of three different extractions A, B and C of GAD according to this protocol are disclosed in Fig. 1. Three Western blots have been carried out for all extractions, namely: 1: cell homogenate; 2: supernatant (after ultracentrifugation); and 3: resuspended pellet (after ultracentrifugation). Purified GAD is used as control. The results clearly and surprisingly show that all GAD is present in the supernatant after centrifugation. The Western blots were performed by using Affiniti antiserum (Affiniti Research Products Ltd., Manhead, Exeter, GB)

EXAMPLE 2

Ion exchange chromatography of extract from baculovirus-infected GAD-producing Sf9 cells

Introduction:

Ion-exchange chromatography (IEX) using Source Q 15 was used for "capture" and first purification of rhGAD65 from extracts of GAD-infected Sf9 cells. Briefly, after extract loading and column washing (10 bed volumes), a gradient to 0.3 M NaCl (over 10 bed volumes) was started, which caused elution of rhGAD65 as a component of the first of two major UV-absorbing peaks at 0.15 M salt. All fractions were collected, aliquots from all fractions removed for analysis to locate eluate fractions with rhGAD65, and all fractions/aliquots were frozen/stored at -

70°C. The aliquots alone (that had been removed from their respective fractions at the time of collection) were then thawed and analysed.

Analyses used to locate rhGAD65 in the eluate fractions collected were the following:

a) total protein components by SDS-PAGE with silver staining. This included both Minigel analyses to identify those fractions with proteins that (when denaturated and run in SDS-PAGE) contain component bands at 65 kDa (see Fig. 5.4), as well as larger SDS-PAGE gels providing better resolution and thereby facilitating the subsequent selection of fractions (Fig. 2).

b) the rhGAD65-specific immunoreactivity by Western blotting using the GAD-6 antibody (Chang and Gottlieb (1988), J. Neuroscience 8(6): 2123-2130) and Affiniti antiserum (Affiniti Research Products Ltd., Manhead, Exeter, GB)(see Fig. 5.6).

c) rhGAD65 enzyme activity.

Subsequently, based on interpretation of these combined data, fractions were selected to maximise GAD concentration (i.e. those with maximal staining intensity of GAD65 band in SDS-PAGE, Western immunoblot, and enzyme activity) while simultaneously minimising protein contaminants. These contaminants were defined as bands visualised on SDS-PAGE gels (e.g. see Fig. 5.5) that do not bind GAD65 antibodies on Western analysis (e.g. see Fig. 5.6). Selected fractions were then thawed, bulked, and immediately refrozen (and stored at -70°C) to provide IEX-purified rhGAD65.

Equipment

Unicorn/FPLC system with P500 pumps and FRAC100

Cold cabinet/cold room 4°C
Pump for packing of column
Column
SOURCE 15Q (Pharmacia Biotech)
Polypropylene fraction collection tubes (sterile, capped)

Chemicals

"Complete" protease inhibitor cocktail tablets (Boehringer Mannheim 1697498)
Pyridoxal-5-Phosphate (Janssen/Chemicon 2281722)
DTT (Sigma D-5545)
Zwittergent 3-12, N-Dodecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate (Boehringer
Mannheim 1112724)
HEPES (Sigma H-3375)
0.5 M EDTA (Sigma E-7889)
NaCl (Merck 1.06404.1000)
NaOH

REAGENTS

1 M NaOH

1 M solution was prepared using strerile distilled water.

I M HEPES stock
(pH 7.2)

119.15 g HEPES was dissolved in 350 ml sterile distilled water. pH was adjusted to 7.2, the volume was adjusted to 500 ml and the solution was passed through a 0.2 Tm sterile filter.

Buffer A

(40 mM HEPES, 5 mM EDTA, 10 mM DTT, 50 TM Pyridoxal-5-phospate, 0.15% Zwittergent 3-12, pH 7.2. Conductivity was ca: 1.2 mS/cm)

Sterile distilled waster	900 ml
1 M HEPES stock	40 ml
0.5 M EDTA	10 ml
DTT	1.5 g
Pyridoxal-5-phospate	13.3 mg
Zwittergent 3-12	1.5 g

pH was adjusted to 7.2 with conc. Hcl. Adjust the volume to 1000 ml.

Buffer B

(was as Buffer A (above) containing 0.3 M NaCl. Condutivity was ca: 20 mS/cm)

NaCl	17.53 g

The buffer was prepared as Buffer A. NaCl was added before adjusting pH and volume.

"Buffer A for sample dilution"

(As Buffer A, plus "Complete" protease inhibitor cocktail)

The buffer was prepared as Buffer A. 3 "Complete" tablets was added for every 125 ml of buffer.

Sample preparation

Immediately before loading the sample (extract) was thawed rapidly by inversion under tap water (40°C), avoiding foaming. The conductivity of the sample was below 4.0 mS/cm.

Flow parameters monitored

The FPLC system included the simultaneous and continuous measurement and display of the following run parameters:

OD_{280}
electrical conductivity
programmed elution gradient
column back-pressure
fraction number

FPLC run protocol

1. Program fraction collector.
2. Fill fraction collector with clean sterile tubes.
3. Equilibrate column with 3 bed volumes Buffer A (at 75 cm/hr).
4. Equilibrate column with 1 bed volume Buffer A (at 60 cm/hr).
5. Apply/load sample (extract) (at 60 cm/hr).
6. Collect flow through for subsequent analysis.
7. Wash for 10 bed volumes (at 60 cm/hr).
8. Collect was eluate for subsequent analysis.
9. Begin linear gradient: 0-100% B over 10 bed volumes at (60 cm/hr).
10. Collect fractions.

11. End method.

Handling of fractions

All tubes used for collection of both flow-through and fractions during gradient elution were sterile and of polypropylene (to minimise breaking during storage below -70°C).

The IEX system used was FPLC (Pharmacia, SE), and the chromatography was largely carried out according to the manufacturer's recommendations.

Fig. 2 discloses the salt gradient part of the obtained chromatogram. The GAD enzyme activity of the eluted fractions were determined according to Blindermann et al (1978)., Eur. J. Biochem. 86:143-152. The results are both presented in the following table, and in Fig. 2.

Sample	Enzyme activity T/ml
55	0.01
59	5.04
61	16.2
63	2.42
65	0.16
69	0.15
71	0.19
75	0
79	0.14
83	0

87	0
91	0.28
97	0

The activity peak in Fig. 2 is situated about two fractions after the main peak. This is due to the amount of eluate between the OD_{280} detector and the fraction collector. Hence, the main peak and the activity peak are closely correlated.

Fig. 3 discloses an SDS-PAGE gel in which lane 1 corresponds to unpurified protein extract and lanes 2-7 correspond to different dilutions of fraction 61 showing the highest degree of purity.

Fig. 4 discloses the result of Western blotting of fractions 55-97. rhGAD65 is only present in fractions 59-63.

Claims

1. Use of a zwitterionic detergent/surfactant for purifying glutamic acid decarboxylase (GAD).

2. A use according to claim 1, **characterized** in that the zwitterionic detergent/ surfactant is a sulphabetaine.

3. A use according to claim 2, **characterized** in that the zwitterionic detergent/ surfactant is a compound according to the following chemical formula:

$$C_yH_{2y+1} - N^+(CH_3)_2 - C_xH_{2x}-SO_3^-$$

wherein x is an integer from 1 to 5 and y is an integer from 2 to 20.

4. A use according to claim 3, **characterized** in that the zwitterionic detergent/ surfactant has a chemical structure chosen from the group of:

$C_8H_{17} - N^+(CH_3)_2 - C_3H_6-SO_3^-$
$C_{10}H_{21} - N^+(CH_3)_2 - C_3H_6-SO_3^-$
$C_{12}H_{25} - N^+(CH_3)_2 - C_3H_6-SO_3^-$
$C_{14}H_{29} - N^+(CH_3)_2 - C_3H_6-SO_3^-$
$C_{16}H_{33} - N^+(CH_3)_2 - C_3H_6-SO_3^-$

5. A use according to claim 4, **characterized** in that the zwitterionic detergent/ surfactant has the chemical structure $C_{12}H_{25} - N^+(CH_3)_2 - C_3H_6-SO_3^-$, such as 3- [(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfone or as 3- [(3-cholamidopropyl)-dimethyl-ammonio]-2-propane-sulfone.

6. A process for extracting glutamic acid decarboxylase from cells containing said enzyme, **characterized** in that the cells are suspended and homogenized in a buffer containing a zwitterionic detergent/surfactant according to anyone of claims 1-5.

7. A process according to claim 6 **characterized** in that the zwitterionic detergent/ surfactant is as 3- [(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfone

8. A process according to claim 6 or claim 7 further comprising an ion exchange chromatography step, wherein all buffers contain a zwitterionic detergent/surfactant according to anyone of claims 1-5.

9. A process according to claim 8, **characterized** in that the zwitterionic detergent is as 3- [(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfone.

10. A process according to anyone of claims 6-9 wherein the concentration of zwitterionic detergent/surfactant in the buffer solutions is within the range of 0,01 - 10% (w/v).

11. A method according to claims 1-10 where identification of eluate fractions containing the baculoviral 65 kDa chitinase protein is used as a control of efficient IEX resolution of 65 kDa proteins in the manufacture of rhGAD.

12. A composition obtainable by a process according to any of claims 6 - 11 comprising glutamic acid decarboxylase and a zwitterionic detergent/surfactant according to any of claims 1 - 4.

13. A composition according to claim 12, **characterized** in that it comprises a zwitterionic detergent/surfactant according to claim 5.

14. A composition according to claims 12-13 for medical use.

15. Use of a composition according to claims 12 - 13 for preparing a pharmaceutical composition for treating IDDM.

16. Use of a composition according to claims 12 - 13 for preparing a pharmaceutical composition for treating cancer.

17. Use of a composition according to claims 12 - 13 for preparing a pharmaceutical composition for treating Stiff Man Syndrome.

18. Use of a composition according to claims 12 -13 for preparing a pharmaceutical composition for treating Graves' Disease.

19.. Use of a composition according to claims 12 - 13 for preparing a pharmaceutical composition for treating autoimmune disease.

20. Use of a composition according to claims 12 - 13 for preparing a vaccine composition against coxsackie virus.

21. Use of a composition according to claims 12 - 13 for preparing a vaccine composition against any of the diseases polio, mumps, parotis and rubella.

22. A kit for detecting antibodies to glutamic acid decarboxylase comprising a composition according to claims 12-13.

23. Use of a composition according to claims 12 - 13 in analytical methods for determining the presence of antibodies to glutamic acid decarboxylase.

Fig. 1

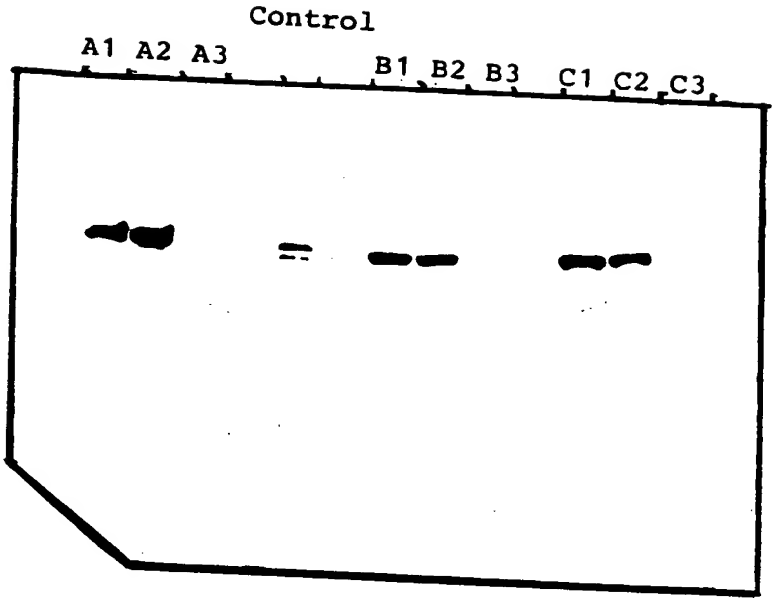


FIG. 2

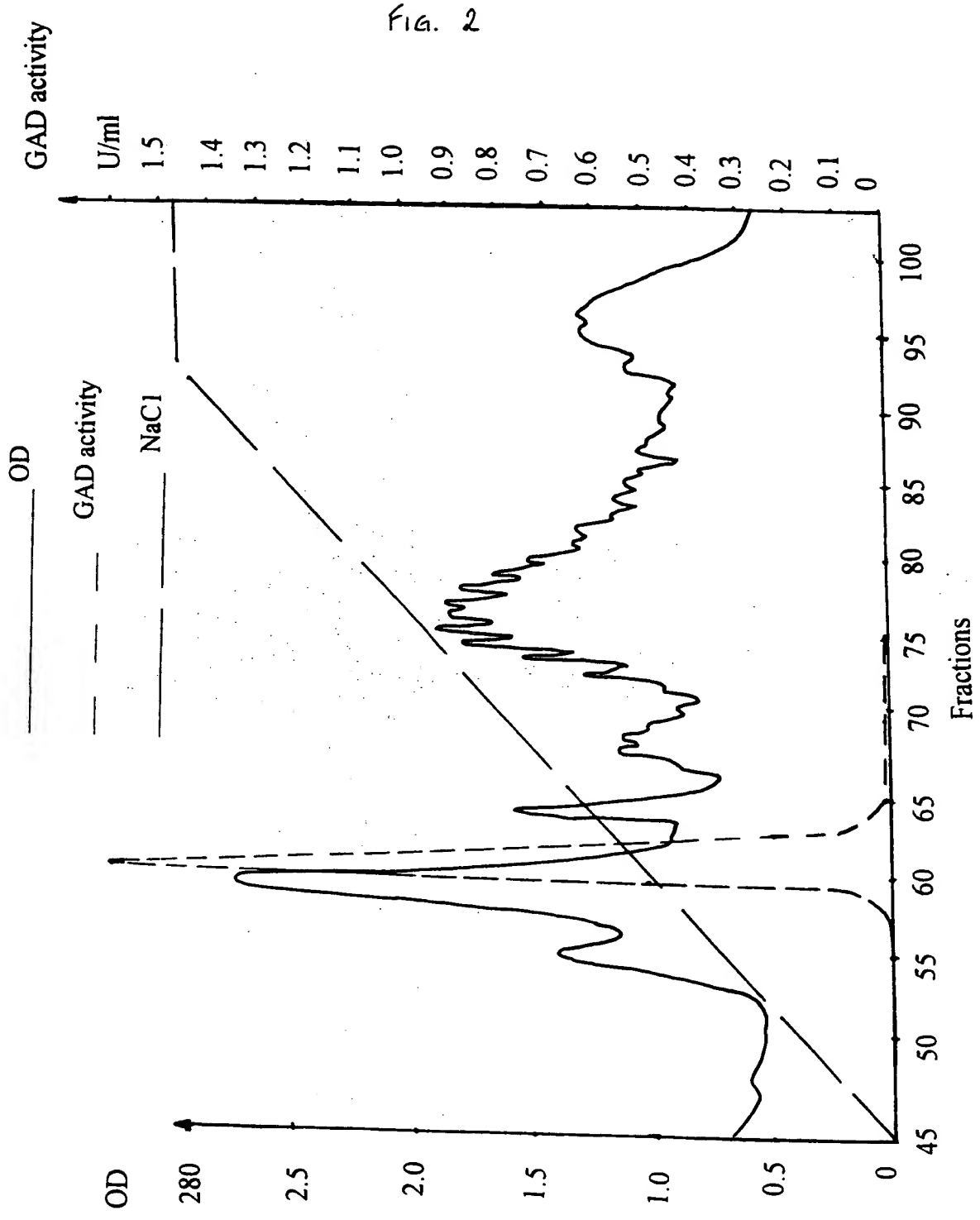

GAD activity
U/ml
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
GAD activity
NaCl
OD
280
2.5
2.0
1.5
1.0
0.5
0
45
50
55
60
65
70
75
80
85
90
95
100
Fractions

Fig. 3

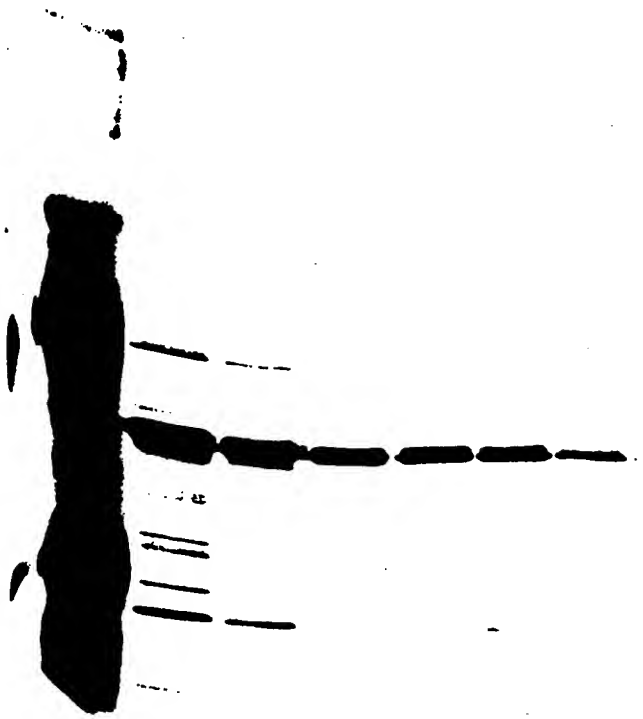
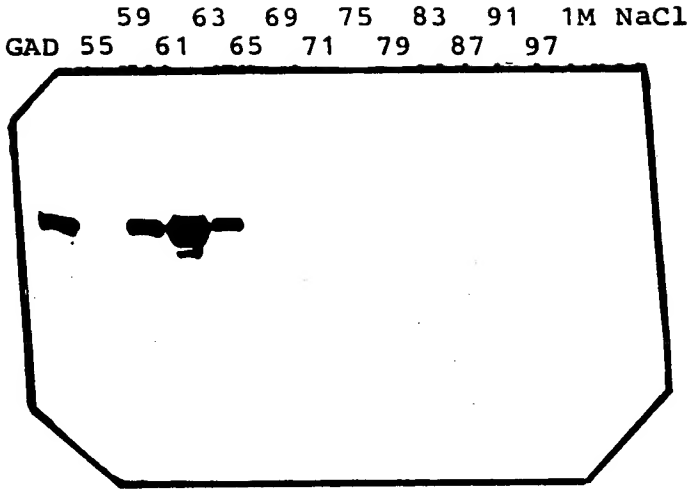


Fig. 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/00723

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/88
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File Medline, Dialog accession no. 07758833, Medline accession no. 94272982, Tursky T. et al: "Glutamic acid decarboxylase in the brain, pancreas and kidneys. Comparison of its properties", Bratisl Lek Listy (SLOVAKIA) Aug 1993, 94 (8) p439-43	1
Y		9-11
A		12-23
	--	
Y	WO 9504137 A1 (NOVO NORDISK A/S), 9 February 1995 (09.02.95), page 3, line 6 - page 4, line 25	1-11
X		12-23
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 Sept 1998	10 -09- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/00723

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Methods in Enzymology, vol. 182, Edited by Murray P. Deutscher, "Guide to Protein Purification", page 505 - page 507; see page 506, line 7-19	1-11
A	Chemical Abstracts, Volume 122, No 11, 12 Sept 1994 (12.09.94), (Columbus, Ohio, USA), Rusell-Harde, Dean, "The use of Zwittergent 3-14 in the purification of recombinant human interferon-beta Ser 17 (Betaseron)", page 850, THE ABSTRACT No 130430p, J. Interferon Cytokine Res. 1995, 15 (1), 31-37	1-11
A	Chemical Abstracts, Volume 106, No 17, 27 April 1987 (27.04.87), (Columbus, Ohio, USA), Wong, Rex K.M. et al, "The efficiency of various detergents for extraction and stabilization of acetylcholinesterase from bovine erythrocytes", page 303, THE ABSTRACT No 134172y, Biochem. Cell Biol. 1987, 65 (1), 8-18	1-11
A	Chemical Abstracts, Volume 103, No 23, 9 December 1985 (09.12.85), (Columbus, Ohio, USA), Karasawa, Ken et al, "Purification and characterization of lysophospholipase L2 of Escherichia coli K-12", page 299, THE ABSTRACT No 192009z, J. Biochem. 1985, 98 (4), 1117-1125	1-11
A	Chemical Abstracts, Volume 95, No 1, 6 July 1981 (06.07.81), (Columbus, Ohio, USA), Bailyes, Elaine M. et al, "The use of a zwitterionic detergent in the solubilization and purification of the intrinsic membrane protein 5'-nucleotidase", page 2382, THE ABSTRACT No 2386r, Biochem. Soc. Trans. 1981, 9 (1), 141-141	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/00723

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Volume 122, No 14, 3 April 1995 (03.04.95), (Columbus, Ohio, USA),page 1239, THE ABSTRACT No 177437m, JP, 6229994 A, (Ko, Fumiji et al) 19 August 1994 (19.08.94) -- --------	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/00723

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No pr test accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE98/00723

The Searching Authority is of the opinion that the present application lacks unity (rule 13.1 PCT) and that in this application two different inventions are claimed for the following reasons:

The first invention claimed relates to a process for purifying glutamic acid decarboxylase by using a zwittericnic detergent /surfactant. (Claims 1-11)

The second invention relates to a composition comprising glutamic acid decarboxylase and a zwitterionic detergent/ surfactant. The composition is used for preparing a pharmaceutical composition for treating different kinds of illness (Claims 12-23)

As there exists no technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features" nonunity exists.

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/07/98

International application No.
PCT/SE 98/00723

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9504137 A1	09/02/95	AU 7226894 A	28/02/95
		EP 0717776 A	26/06/96